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(54) Title: VACCINES FOR EQUINE HERPESVIRUS TYPE-1: VIRUSES HARBORING MUTATIONS WITHIN THE IMMEDIATE EARLY GENE

(57) Abstract: This invention relates to mutant Equine herpesvirus type-1 (EHV-1) isolates, and in particular, EHV-1 isolates carrying mutations in the immediate-early (IE) gene of the viral genome. A panel of EHV-1 mutant isolates have been described. Preferred EHV-1 isolates are those which are replication-competent and nonpathogenic. The mutant EHV-1 isolates of the present invention are useful in formulating vaccine compositions for preventing and treating EHV-1 infections in horses. The present invention further provides methods of determining the pathogenicity of an EHV-1 virus present in a horse subject which has been previously immunized with a non-pathogenic EHV-1 isolate of the present invention.

5                   **Vaccines For Equine Herpesvirus Type-1:**  
                  **Viruses Harboring Mutations Within the Immediate Early Gene**

10                   This invention relates to mutant Equine herpesvirus  
                  type-1 (EHV-1) isolates, and in particular, EHV-1 isolates  
                  carrying mutations in the sole immediate-early (IE) gene of  
                  the viral genome. The present invention further relates to  
                  vaccine compositions and methods of treating EHV-1 infections.  
15                   Methods of determining the pathogenicity of an EHV-1 virus are  
                  also provided.

                  Equine herpesvirus type-1 (EHV-1) is a major  
                  pathogen in horses. In infected animals, it is usually  
20                   associated with upper respiratory tract infections, but may  
                  also cause neurological sequelae. EHV-1 infection in pregnant  
                  mares causes abortigenic disease and has an important economic  
                  impact on the worldwide equine industry.

                  Although the horse is the natural host of the equine  
25                   herpesviruses, a variety of animals and tissue culture systems  
                  can be used to propagate the viruses. Experimental animals  
                  for EHV-1 include Syrian hamsters, baby hamsters, chick  
                  embryos, baby and adult mice, and kittens. Primary tissue  
                  culture systems used to propagate EHV-1 include cells from a  
30                   variety of equine tissues such as fetal lung, dermis, spleen  
                  and kidney, as well as cells from domestic cats, dogs,  
                  hamsters, rabbits, mice, sheep and swine. In the laboratory,

permanent tissue culture systems commonly used to cultivate EHV-1 include HeLa, Vero, CV-1, rabbit kidney (RK), mouse L-M and equine Edmin337 cells.

Natural respiratory EHV-1 infection of the horse  
5 only results in a short-lived humoral response and does not confer long-term protection against subsequent infection. A number of vaccines have been developed to combat EHV-1 infections, among them inactivated vaccines which mostly contain both EHV-1 and EHV-4 (Pneumabort K™, Resequin™,  
10 Prestige™ and Duvaxyn™), modified live vaccines (RhinoMune™ or Prevaccinol™) and subunit vaccines (Cavalon IR™). However, some EHV vaccines cause undesirable side effects, and most do not afford acceptable levels of protection. There is a need for safe and effective vaccines against EHV-1  
15 infection.

The present invention is directed to mutant equine herpesvirus type-1 (EHV-1) isolates carrying one or more mutations in the immediate-early (IE) gene of the viral  
20 genome.

In particular, the present invention provides EHV-1 isolates carrying in the IE gene of the viral genome, at least one of the mutations listed in Table 1.

In a preferred embodiment, the present invention  
25 provides replication-competent EHV-1 isolates carrying one or more mutations in the IE gene.

In particular, the mutation in the IE gene does not significantly interfere with the structure and/or function of any of the four domains of the IE protein, TAD (aa 1-89), SRT  
30 (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970), which are essential for viral replication.

In this embodiment, the present invention provides replication-competent EHV-1 isolates which carry a mutation in the IE gene that involves a substitution of an amino acid residue within TAD, SRT, DBD or NLS. Preferably, the  
5 substitution involves an exchange of amino acids within an exchange group, i.e., amino acids that resemble each other with respect to their overall impact on protein structure. More preferably, the substitution is one of D24N, D20N, F15D, L12E or E34Q.

10 Particularly preferred mutations include insertions or deletions of one or more, preferably, at least three, more preferably at least five, amino acid residues within amino acid 90-180, 221-421, 598-962, or 971-1487. Most preferred mutations include d644/824 (deletion of aa 644-824), n1029  
15 (nonsense mutation at 1029 and deletion of aa 1030-1487), n1411 (nonsense mutation at 1411 and deletion of aa 1411-1487), in628 (insertion at 1411) and in1411 (insertion at 1411).

In a preferred embodiment, the present invention  
20 provides replication-competent EHV-1 isolates which carry one or more mutations in the IE gene and which have reduced virulence or no virulence, also referred herein as "non-pathogenic" EHV-1 isolates.

Non-pathogenic, replication competent EHV-1 isolates  
25 can be of an EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM wherein one or more mutations have been introduced into the IE gene of the viral genome. Preferred mutant EHV-1 isolates are KyA mutant isolates.

The present invention is further directed to  
30 immunogenic compositions which include one or more of the non-

pathogenic, replication-competent mutant EHV-1 isolates described herein.

The present invention further provides methods of stimulating an immune response against EHV-1 in a horse subject by administering to the horse an immunogenic composition of the present invention.

Still further the present invention is directed to live attenuated vaccine compositions which include one or more of the non-pathogenic, replication-competent mutant EHV-1 isolates described herein.

Still even further, the present invention provides methods of treating EHV-1 infections in a horse by administering to the horse subject, a therapeutically effective amount of a non-pathogenic, replication-competent mutant EHV-1 isolate of the present invention.

Yet even further, the present invention provides methods for determining the pathogenicity of an EHV-1 strain present in a subject previously vaccinated with a non-pathogenic EHV-1 isolate of the present invention. The determination is achieved by carrying out assays which distinguish the wild type EHV-1 and the non-pathogenic EHV-1 isolate previously administered to the subject.

25

Figure 1 depicts the structure of the EHV-1 genome and location of the immediate-early gene. A schematic of the EHV-1 genome is shown at the top. The lower portion of the figure depicts the functional domains of the IE protein (1,487 amino acids): the transactivation domain (TAD, aa 3-89), a serine rich tract (SRT, aa 131-220), the DNA-binding domain

30

(DBD, aa 422-597), nuclear localization signal (NLS, aa 963-970).

Figure 2 depicts the recombination system to generate mutations in the IE gene. *Left panel:* Construct pIECassette contains the 5' portion of the IE gene. Unique NcoI and NaeI restriction sites facilitate the cloning of sequences encoding mutagenized TADs spanning amino acids 3-89. *Right panel:* Construct pBR322IE contains the entire IE ORF. EcoRV/BamHI fragments from various pIECassette constructs containing mutagenized TADs were easily cloned into pBR322IE to reconstitute an ORF that encodes a mutant form of the IE protein. Alternatively, various domains were swapped, replacing the wild-type sequences with various mutagenized sequences. This vector was used to generate recombinant viruses. Recombination plasmids were transfected into IE13.1 cells which were superinfected with KyAΔIE at 24 h post-transfection. Supernatants were screened on RK-13 cells for viruses that were able to propagate in the absence of complementing IE protein.

Figure 3 depicts the Western analyses of EHV-1 mutants. *Panel A*, infected-cell extracts (ICE) derived from RK-13 cells infected with EHV-1 KyA (Lane 1), KyAd644/824 (Lane 2), KyAn1411 (Lane 3), or KyAIn1411 (Lane 4) at an MOI of 10. ICE were subjected to SDS-PAGE, and the proteins were blotted to nitrocellulose and were stained with the polyclonal anti-IE peptide antibody. The 200-kDa band representing the IE protein was clearly detected in Lanes 1, 3, and 4. The 175-kDa band derived from KyAd644/824-infected cells is shown in Lane 2. *Panel B*, Lane 1 shows the 200-kDa IE protein band detected in nuclear extracts of RK-13 cells infected with EHV-

1 KyA. Lane 2 shows the 138 kDa IE protein band detected in nuclear extracts of RK-13 cells infected with KyAn1029.

Figure 4 depicts the growth analyses of selected IE mutant viruses. RK-13 cells were infected with either wild-type EHV-1 KyA or selected EHV-1 IE mutant viruses at an MOI  
5 of 1 and incubated for 1 h at 37°C in 5% CO<sub>2</sub> to permit virus attachment. After attachment, the cells were washed three times with Eagle's without FBS to remove unattached virus and were incubated at 37°C in 5% CO<sub>2</sub>. At the times indicated after  
10 the attachment period, virus titers in the culture supernatant were determined by plaque assay using RK-13 cells.

Figure 5A-5C depict the analysis of the CTL activity of the lymphocytes isolated from CBA mice immunized with wild type EHV-1 or EHV-1 containing mutations in the IE gene.  
15

One aspect of the present invention is directed to novel mutant equine herpesvirus type-1 (EHV-1) isolates carrying one or more mutations in the immediate-early (IE) gene of the viral genome.

20 EHV-1 has a linear, double-stranded DNA genome, characterized by short and long unique sequences (U<sub>S</sub> and U<sub>L</sub> respectively), and inverted repeats which flank the unique short sequences. The entire genome of EHV-1, strain Ab4, has been sequenced and shown to be 150,223 bp in size and contain  
25 80 potential open reading frames (ORFs).

The IE gene is the sole immediate-early gene of EHV-1 and is present in both inverted repeats of the viral genome. The open reading frame (ORF) of the IE gene (SEQ ID NO: 1) is transcribed to a 6.0-kb spliced mRNA that gives rise to both  
30 structurally and antigenically-related protein species

(Caughman et al. Virology 163:563-571, 1988). The predominant IE protein species of 1,487 amino acids (SEQ ID NO: 2) is comprised of discrete, functional domains (Figure 1). A potent transcriptional activation domain (TAD) maps within the first 89 amino acids. A serine rich tract (SRT; aa 181-220) may contain a site(s) for phosphorylation. The DNA-binding function lies within amino acid residues 422-597. Amino acids spanning 963-970 (NLS) are necessary for nuclear localization of the protein.

The IE protein is essential for viral growth in cell culture and is required for both early and late gene expression during the course of a productive infection (Smith et al. J. Virol. 66:936-945, 1992). Following IE polypeptide synthesis, approximately 45 early transcripts can be detected. Three of these early proteins serve as regulatory proteins and are designated EICP22, EICP27 and EICP0. Early gene expression is followed by the production of approximately 29 late transcripts and viral replication, after which mature virions are generated.

The term "mutation" as used herein includes substitution, deletion or insertion of one or more base pairs in the IE coding sequence which results in a substitution, deletion or insertion of one or more amino acid residues in the IE protein.

According to the present invention, EHV-1 isolates carrying mutations in the IE gene can be generated by a recombination system provided herein. In accordance with such system, mutations in the IE gene can be generated by employing any of the myriad recombinant cloning techniques, e.g., those described in *Current Protocols in Molecular Cloning* (Ausubel et al., John Wiley & Sons, New York). A mutant IE nucleotide



sequence carrying one or more mutations is then placed on a recombination vector appropriate for transfection and transfected into an appropriate host cell, e.g., RK-13 cells. Transfected host cells are then infected with a null EHV-1 virus devoid of the IE gene. Mutant viruses are thus generated by homologous recombination between the genome of the null virus and the recombination vector containing the mutant IE gene. Null EHV-1 viruses for use in this recombination system can be generated using any EHV-1 strain, e.g., KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. A deposit of the EHV-1 KyA strain was made with the American Tissue Type Culture, 10801 University Blvd., Manassas, VA 20110-2209, on July 20, 2000 (ATCC deposit # PTA-2253). Null virus of strain KyA, i.e., KyAΔIE, has been generated as described by Garko-Buczynski et al. (Virology 248: 83-94, 1998). By way of this recombination system, mutant EHV-1 isolates carrying identical or different mutations in the two copies of the IE gene can be generated. Preferably, the mutant EHV-1 isolates of the present invention carry identical mutation(s) in both copies of the IE gene in the viral genome.

One embodiment of the present invention provides EHV-1 isolates carrying in the IE gene of the viral genome, at least one of the mutations listed in Table 1.

Table 1

Deletion Mutations	Nature of Mutation	Domain Affected
ΔSRT1	aa 181-250 deleted	Deletion of SRT
ΔSRT2	aa 38-243 deleted	Deletion of SRT
d178/627	aa 178 to 627 deleted	Deletion of SRT and DBD
d552/897	aa 552 to 897 deleted	Deletion of part of DBD

d644/824 aa 644 to 824 deleted

#### Nonsense Mutations

n627	aa 628-1487 deleted	Deletion of NLS and C-term
n951	aa 952-1487 deleted	Deletion of NLS and C-term
n1029	aa 1030 -1487 deleted	Deletion of C-term
n1411	aa 1412 - 1487 deleted	Deletion of C-term

#### Insertion Mutations

in623	insertion at aa 628
in1411	insertion at aa 1411

#### Point Mutations: Amino Acid Substitutions

D24N	Asp 24 to Asn	Point mutation in TAD
D20N	Asp 20 to Asn	Point mutation in TAD
F15D	Phe 15 to Asp	Point mutation in TAD
L12P	Leu 12 to Pro	Point mutation in TAD
L12E	Leu 12 to Glu	Point mutation in TAD
E34Q	Glu 34 to Gln	Point mutation in TAD

In a preferred embodiment, the present invention provides mutant EHV-1 isolates that are replication-competent.

The term "replication competence" as used herein refers to the ability of a viral isolate to propagate in a host cell in the absence of a complementing IE protein expressed in *trans*.

According to the present invention, the replication competence of a mutant viral isolate can be determined by a number of assays, e.g., a plaque assay using non-complementing cells (cells that do not express IE protein). In this assay, cells of a monolayer are infected with a mutant isolate of interest and are subsequently overlaid with 2% agarose mixed in a 1:1 ratio with appropriate growth medium. Host cells which can be employed for this purpose include cells from a

variety of equine tissues such as fetal lung, dermis, spleen and kidney, as well as cells from domestic cats, dogs, hamsters, rabbits, mice, sheep and swine. Typically, cells commonly used to cultivate EHV-1 in tissue culture are used, including HeLa, Vero, CV-1, rabbit kidney (RK), mouse L-M and equine Edmin337 cells. Preferably, RK-13 cells are used in the assay.

A mutant viral isolate is "replication competent" if such mutant isolate can form plaques on the non-complementing cells, even if the plaques may be of a smaller size than those formed by wild type EHV-1. Replication-incompetent cells can only form plaques in complementing cells where the IE protein is expressed in trans e.g., IE13.1 cells.

According to the present invention, four domains of the IE protein, TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970), are essential for viral replication. Thus, mutations in the IE gene that significantly interfere with the structure and/or function of these four domains likely produce replication incompetent viral isolates. For example, a deletion of five or more contiguous amino acid residues within any of these four domains of the IE protein is likely disruptive to the function of such domain, and the resulting mutant virus is likely to be replication-incompetent. However, substitution of one or more amino acid residues to residues similar in size and/or hydrophobicity can be less disruptive to the structure and/or function of the respective domain and thus, the resulting mutant virus can still be replication-competent.

Accordingly, one embodiment of the present invention provides replication-competent EHV-1 isolates harboring at least one mutation in the IE gene, wherein the mutation is a

substitution at a residue anywhere within TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) or NLS (aa 963-970) and wherein the mutation does not substantially disrupt the function of the IE protein in viral replication.

5           Although not limited to any specific formula, a preferred substitution according to the present invention can be between amino acids within an exchange group, i.e., amino acids that resemble each other with respect to their overall impact on protein structures. For example, aromatics Phe, Tyr  
10 and Typ form an exchange group; the positively charged residues Lys, Arg and His form an exchange group; the large aliphatic non-polar residues Val, Leu and Ile form an exchange group which also contains the slightly polar Met and Cys. All small residues Ser, Thr, Asp, Asn, Gly, Ala, as well as Glu,  
15 Gln and Pro are also within an exchange group.

More preferably, the substitution is one of D24N, D20N, F15D, L12E or E34Q. Even more preferably, the mutant EHV-1 isolates carrying one or more of these substitutions are of a strain selected from KyA, KyD, Abl, Ab4, RacL11, RacM or  
20 RacH. Particularly preferred mutant EHV-1 isolates are KyAD24N, KyAD20N, KyAF15D, KyAL12E and KyAE34Q.

In another preferred embodiment of the present invention, the replication-competent mutant EHV-1 isolates harbor a mutation that is localized outside of any of the four  
25 domains described above and does not cause any substitution, deletion or insertion within any of the TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970) domains.

Particularly preferred mutations include insertions or deletions of one or more, preferably, at least three, more  
30 preferably at least five, amino acid residues within the region of amino acid 90-180, 221-421, 598-962, or 971-1487.

Most preferred mutations include d644/824 (deletion of aa 644-824), n1029 (nonsense mutation at 1029 and deletion of aa 1030-1487), n1411 (non-sense mutation at 1411, and thus deletion of aa 1411-1487), in628 (insertion at 1411) and  
5 in1411 (insertion at 1411). The mutant EHV-1 isolates carrying one or more of these mutations are preferably of a strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH, and more preferably, KyA.

In a preferred embodiment, the present invention  
10 provides replication-competent mutant EHV-1 isolates that have reduced virulence or no virulence, also referred herein as "non-pathogenic" or "attenuated" EHV-1 isolates.

The term "virulence" or "pathogenicity" as used herein refers to the capacity of a strain of EHV-1 to induce  
15 EHV-1-related diseases in horses, e.g., infection in the respiratory tracts, spontaneous abortions as well as neurological diseases. Infections caused by pathogenic EHV-1 are typically characterized by fever, profuse nasal discharge and congestion of the nasal mucosa. Accordingly, a "non-  
20 pathogenic" or "attenuated" strain of EHV-1, or an EHV-1 strain with "reduced virulence" as used herein is a strain having a substantially reduced capacity, as compared to a pathogenic EHV-1, in inducing the development of EHV-related clinical diseases.

25 According to the present invention, the pathogenicity of an EHV-1 isolate can be conveniently determined in mouse models. Clinical signs of EHV-1 infection in mice include, e.g., ruffled fur, loss of body weight, labored breathing, lethargy and huddling, as described by,  
30 e.g., Colle et al. Virus Res. 43: 111-124 (1996) and Zhang et al. Virus Res. 56: 11-24 (1998). In addition, the degree of

infection can also be assessed by isolating the viruses from the lungs of infected mice, plating the viruses on RK-13 cell monolayers and determining the number of plaques formed, as described in the Examples that follow.

5           The non-pathogenic mutant EHV-1 isolates of the present invention can be generated by introducing a mutant IE gene into null viruses of a non-pathogenic EHV-1 strain via the recombinant system described herein. Any of those naturally non-pathogenic EHV-1 strains or EHV-1 strains that  
10   are made non-pathogenic by laboratory procedures can be used for generating null-viruses, e.g., KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Null viruses of any of these non-pathogenic EHV-1 strains can be generated, e.g., by following the procedure described by Garko-Buczynski et al. (Virology 248:  
15   83-94, 1998).

A preferred EHV-1 strain for use in generating the mutant isolates of the present invention is the EHV-1 KyA strain, a deposit of which was made with the American Tissue Culture Type Culture, 10801 University Blvd., Manassas, VA 20110-2209,  
20   on July 20, 2000 (ATCC deposit # PTA-2253).

Accordingly, preferred non-pathogenic, replication competent mutant EHV-1 isolates of the present invention include KyA isolates carrying at least one mutation in the IE gene, wherein the mutation is a substitution at a residue  
25   anywhere within TAD (aa 1-99), SRT (aa 181-220), DBD (aa 422-597) or NLS (aa 963-970) and wherein the mutation does not substantially disrupt the function of the IE protein in viral replication. Examples of such mutant EHV-1 isolates are KyAD24N, KyAD20N, KyAF15D, KyAL12E and KyAE34Q.

30           Other preferred non-pathogenic, replication competent mutant EHV-1 isolates of the present invention

include KyA isolates carrying at least one mutation in the IE gene, wherein the mutation is localized outside of any of the four domains described above and does not cause any substitution, deletion or insertion within any of the TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970) domains. Examples of such mutant EHV-1 isolates are KyAd644/824, KyAn1029, KyAn1411, KyAin628 and KyAin1411.

In a further aspect of the invention, the non-pathogenic, replication-competent mutant EHV-1 isolates of the present invention as described hereinabove are included in immunogenic compositions.

By "immunogenic" is meant the capacity of an EHV-1 isolate in provoking a immune response in a horse subject, either a cellular immune response mediated primarily by cytotoxic T-cells, or a humoral immune response mediated primarily by helper T-cells which in turn activate B-cells leading to antibody production.

The immunogenic compositions of the present invention include at least one, i.e., one or more of the non-pathogenic replication competent mutant EHV-1 isolates described hereinabove. Preferred mutant EHV-1 isolates to be included in the immunogenic compositions include, e.g., an EHV-1 isolate harboring one or more of the mutations D24N, D20N, F15D, L12E or E34Q in the IE gene, or an EHV-1 isolate harboring one or more mutations within aa 90-180, 221-421, 598-962 or 971-1487 of the IE protein. Preferably, the mutant EHV-1 isolates for use in an immunogenic composition of the present invention are of a non-pathogenic EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Particularly preferred mutant EHV-1 isolates for use in an immunogenic

composition include KyAD24N, KyAD20N, KyAF15D, KyAL12E, KyAE34Q, KyAd644/824, KyAn1411 and KyAin1411.

The immunogenic compositions of the present invention can also include additional active ingredient such as other immunogenic compositions against EHV-1, e.g., those described in U.S. Patent 5,707,629 ("Immunogenic composition against equine herpesvirus type 1") and U.S. Patent 5,795,578 ("Vaccine against equine herpesvirus type 1), or immunogenic compositions against EHV-4.

In addition, the immunogenic compositions of the present invention can include one or more pharmaceutically-acceptable carriers.

As used herein "a pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, adjuvant, vaccine stabilizing agents, antibacterial and antifungal agents, isotonic agents such as sugar and sodium chloride, adsorption delaying agents, and the like. The use of such media gents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the immunogenic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The immunogenic compositions of the present invention can made in forms suitable for injectable use, e.g., in the form of sterile aqueous solutions or dispersions, or can be made in lyophilized forms using vacuum-drying and freeze-drying techniques. Lyophilized vaccine compositions are typically maintained at about 4°C, and can be reconstituted in a stabilizing solution, e.g., saline or and



HEPES, with or without adjuvant. In all cases the form of the immunogenic compositions must be sterile.

The immunogenic compositions of the present invention can be administered to a horse to induce an immune response against EHV-1. Accordingly, another embodiment of the present invention provides methods of stimulating an immune response against EHV-1 in a horse subject by administering an effective amount of any one of the above-described immunogenic compositions of the present invention.

The amount of an immunogenic composition to be administered to be "effective" in inducing an immune response may depend on the immunogenicity of the particular EHV-1 isolate used in the immunogenic composition. According to the present invention, the immunogenicity of an EHV-1 isolate, i.e., the type and extent of an immune response induced by a viral isolate can be conveniently assessed in mice, which are generally accepted as models representative of horse subjects. A variety of techniques may be used for analyzing the immune responses induced in mice by a viral isolate. For example, one skilled in the art can determine whether a viral isolate induces a cell-mediated immune response by, e.g., detecting the presence of responding CTLs in the mouse spleen or other lymphoid tissues. One skilled in the art can also readily determine whether a viral isolate stimulates a humoral immune response by, e.g., detecting the neutralizing titer of EHV-1 specific antibody in the serum or the presence of antibody secreting cells in the serum. These techniques are well described in the art, e.g., Coligan et al. Current Protocols in Immunology, John Wiley & Sons Inc. (1994).

For inducing an immune response, an immunogenic composition of the present invention can be administered to a

horse subject via intravenous, intraperitoneal, intramuscular, or intramucosal (e.g. nasal or respiratory spray or injection) routes, or by other forms of parenteral administration. An immunogenic composition can also be administered via an  
5 implant or orally.

Another embodiment of the present invention is directed to live attenuated vaccine compositions.

More specifically, the vaccine compositions of the present invention include one or more of the non-pathogenic, replication-competent mutant EHV-1 isolates as described  
10 hereinabove.

The infection of a cell or cells by a pathogenic strain of EHV-1 leads to the production of pathogenic virions in the infected subjects and the EHV-1 related diseases. In  
15 contrast, a non-pathogenic EHV-1 strain of the present invention generally replicates to an extent to sufficient to protect the subject against challenge by a virulent or pathogenic EHV-1 strain.

The term "vaccine" as used herein refers to a  
20 composition which prevents or reduces the risk of infection or which ameliorates the symptoms of infection. The protective effects of a vaccine composition against a pathogen are normally achieved by stimulating an immune response in the subject which may involve either or both of cell-mediated or  
25 humoral immune response. The strength and duration of the immune responses induced by an EHV-1 isolate can be taken into consideration in determining the amount of such isolate that should be included in a vaccine composition, as well as the vaccination schedules. Generally speaking, abolished or  
30 reduced incidences of EHV-1 infection, amelioration of the symptoms, or accelerated elimination of the viruses from the

infected subjects are indicative of the protective effects of a vaccine composition.

Preferred non-pathogenic, replication competent mutant EHV-1 isolates which can be used in a vaccine composition include, e.g., an EHV-1 isolate harboring one or more of D24N, D20N, F15D, L12E or E34Q in the IE gene. Preferably, the mutant EHV-1 isolate for use in a vaccine composition is of a non-pathogenic EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Particularly preferred EHV-1 isolates for use in a vaccine composition include KyAD24N, KyAD20N, KyAF15D, KyAL12E and KyAE34Q.

Other preferred non-pathogenic, replication competent mutant EHV-1 isolates which can be used in a vaccine composition include an EHV-1 isolate harboring one or more mutations within aa 90-180, 221-421, 598-962, or 971-1487 of the IE protein, for example, d644-824, n1411 and in1411. Preferably, the mutant EHV-1 isolate for use in a vaccine composition is of a non-pathogenic EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Particularly preferred EHV-1 isolates for use in a vaccine composition include KyAd644/824, KyAn1411 and KyAin1411.

The vaccine compositions of the present invention can also include additional active ingredient such as other immunogenic compositions against EHV-1, e.g., those described in U.S. Patent 5,707,629 ("Immunogenic composition against equine herpesvirus type 1") and U.S. Patent 5,795,578 ("Vaccine against equine herpesvirus type 1), or immunogenic compositions against EHV-4.

Furthermore, the vaccine compositions of the present invention can include one or more pharmaceutically-acceptable carriers as described hereinabove.

The vaccine compositions of the present invention can made in forms suitable for injectable use, e.g., in the form of sterile aqueous solutions or dispersions, or can be made in lyophilized forms using vacuum-drying and freeze-drying techniques. Lyophilized vaccine compositions are typically maintained at about 4°C, and can be reconstituted in a stabilizing solution, e.g., saline or and HEPES, with or without adjuvant. In all cases the form of the vaccine compositions must be sterile.

In another embodiment of the present invention, the above vaccine compositions of the present invention are used in treating EHV-1 infections. Accordingly, the present invention provides methods of treating EHV-1 infections in a horse by administering to the horse subject, a therapeutically effective amount of a non-pathogenic EHV-1 isolate of the present invention.

By "treating" is meant preventing or reducing the risk of infection by a pathogenic strain of EHV-1, ameliorating the symptoms of an EHV-1 infection, or accelerating the recovery from an EHV-1 infection.

The amount of a non-pathogenic EHV-1 isolate that is therapeutically effective may depend on the nature of the isolate, the condition of the horse and/or the degree of infection, and can be determined by a veterinary physician.

In practicing the present methods, a vaccine composition of the present invention can be administered to a horse subject via intravenous, intraperitoneal, intramuscular, or intramucosal (e.g. nasal or respiratory spray or injection) routes, or by other forms of parenteral administration. A vaccine composition can also be administered via an implant or orally. Boosting regiments may be required and the dosage

regimen can be adjusted to provide optimal immunization. The vaccination of a mare prior to breeding and again during her pregnancy may prevent abortions caused by EHV-1. Other horses can be vaccinated, for example, about once a year. Foals can  
5 be vaccinated shortly after birth.

Non-pathogenic, replication competent mutant EHV-1 isolates of the present invention can also have immune protective effects against infections caused by equine herpesvirus type 4 (EHV-4). Thus, the use of the mutant EHV-1  
10 isolates of the present invention in immunogenic or vaccine compositions for preventing or inhibiting EHV-4 infections is also contemplated by the present invention.

The vaccine compositions of the present invention have the additional feature that the non-pathogenic EHV-1  
15 isolate included therein is generally discernable from wild type EHV-1 strains in terms of the composition and size of the IE protein expressed, or the sensitivity of growth to temperature. This feature is useful, e.g., in determining whether a subject tested positive for EHV-1 in certain  
20 laboratory tests carries a pathogenic EHV-1 or a previously inoculated non-pathogenic EHV-1.

Accordingly, in another embodiment, the present invention provides methods of determining the pathogenicity of an EHV-1 strain present in a subject previously vaccinated  
25 with a non-pathogenic EHV-1 isolate of the present invention.

As the methods are premised on a distinction between the wild type EHV-1 and the non-pathogenic EHV-1 isolate previously administered to the subject, the choice of assays for making the distinction depends on the nature of the  
30 mutation of the non-pathogenic EHV-1 isolate.

When the non-pathogenic viral isolate previously administered to the subject contains a deletion in the IE gene, EHV-1 having d644/824, n1029 or n1411, assays based on detection of IE proteins or antibodies in serum against IE proteins can be employed. For example, infected cells or tissues can be isolated from the subject. Intracellular extracts can be made from such cells or tissues and can be subjected to, e.g., Western Blot analysis, as described in the Examples hereinbelow. The observation of an IE protein of a lower Mw is indicative of the non-pathogenicity of the EHV-1 strain present in the subject. Alternatively, an antibody specific for the deleted portion of amino acid residues can be used in Western Blot, and absence of an IE protein band is also indicative of the non-pathogenicity of the EHV-1 strain present in the subject. Additionally, the absence of antibodies in the serum against the deleted or truncated portion of the IE protein, may also be an indication of the non-pathogenicity of the virus in the subject. The presence or absence of antibodies in the serum of the subject can be determined by using a peptide corresponding to the deleted portion in an appropriate immunoassay, e.g., ELISPOT.

When the non-pathogenic viral isolate previously used in vaccination has a different sensitivity to temperature as compared to wild type EHV-1, temperature shift assays can also be used, as described in the Examples hereinbelow. For example, such assays can be applied in distinguishing wild type EHV-1 from, e.g., EHV isolates having d644/824, n1029, n1411, in1411, D24N, L12E, F15D or E34Q, and in particular, n1029 and n1411. Unlike wild type KyA, KyAn1411 and KyA1029 fail to grow at 39 C. KyA carrying d644/824, in1411, D24N,

L12E, F15D or E34Q has a reduced capacity to grow as compared to wild type KyA.

The determination of the pathogenicity can also be achieved by using nucleic acid-based assays to screen for mutations in the IE gene of the viruses isolated from the subject. These assays include Southern or Northern blot analysis, PCR, and sequencing.

A mutation in the IE gene may result in a reduced expression of another EHV-1 protein, e.g., EICP0 or gD. For example, n1029 causes significant reduction in the mRNA levels of both EICP0 and gD. In this case, detection of the reduced expression of such other proteins, either at the mRNA level or the protein level is indicative of the non-pathogenicity of the virus in the subject.

Any variations of the foregoing assays are also encompassed by the present invention.

The present invention is further illustrated by the following examples.

Example 1**Materials and Methods**

5        Mouse LM cells were propagated in suspension culture  
with YELP medium (Eagle's minimum essential medium [EMEM]  
supplemented with yeast extract, lactalbumin hydrolysate,  
peptone) containing 0.12% methylcellulose-12, 100 µg/ml  
streptomycin, 100 U/ml penicillin, and 5% fetal bovine serum  
(FBS).

10        Rabbit kidney cells (RK-13) were maintained in  
complete EMEM supplemented with 100 µg/ml streptomycin, 100  
U/ml penicillin, nonessential amino acids and 5% FBS.  
Infectious virus was measured by a plaque assay as described  
by Perdue et al. Virology 59: 201-216 (1974).

15        IE13.1 cells express the IE protein in *trans* and  
both copies of the IE gene on the viral genome are replaced  
with LacZ sequences from *E. coli*. Generation and  
characterization of IE13.1 cells as well as the IE knock-out  
virus KyAΔIE were described by Garko-Buczynski et al. Virology  
20    248: 83-94 (1998).



## Example 2

### Generation of Mutant Viruses

#### Plasmids

5           A recombination system was established (Figures 1-2) so that a panel of mutant viruses could be generated by homologous recombination between the KyAAIE viral genome and various recombination vectors derived from plasmid pBR322IE.

          To generate the first recombination vector pBR322IE,  
10 pBR322 was digested with *EcoRV* and *PvuII*, and the resulting fragment was re-ligated to generate plasmid pBR322E/P. An 8.0-kb *NdeI* fragment containing the entire IE ORF from nt -215 to nt +8140 of the IE gene (Grundy et al., Virology 172: 223-236, 1989) was cloned into the sole *NdeI* site of pBR322E/P to  
15 generate pBR322IE. A second vector, pIECassette, was generated by cloning an *EcoRV/BamHI* fragment that harbors nt -257 to +1266 of the IE ORF into the *EcoRV/BamHI* sites of pBR322.

          A "domain swap" strategy was used to generate  
20 recombination plasmids containing mutant IE forms with nonsense, insertion, point, or deletion mutations. Recombination plasmids pBR322n1411, pBR322d644/824, pBR322In628, and pBR322n1029 were generated by digesting pBR322IE with *BamHI* and *PvuII* and replacing this fragment with  
25 mutagenized *BamHI-PvuII* IE ORF sequences derived from mutants described by Smith et al. J. Virol. 69: 3857-3862 (1995). Plasmids pBR322d178/627 and pBR322d552/897 were generated by digesting pBR322IE with *NcoI* and *PvuII* and replacing this  
30 fragment with mutagenized *NcoI-PvuII* IE ORF sequences derived from mutants described by Buczynski et al., Virus Res. 65: 131-140 (1995) and by Caughman et al. J. Virol. 69: 3024-3032

(1995). Recombination plasmid pBR322 $\Delta$ SRT1 was generated by digestion of pSVIE with *BspEI* (nt 1523 to nt 1733) followed by fill-in with Klenow to generate blunt-ends and insertion of an in-frame 10-mer *EcoRI* linker. To construct the pBR322IE $\Delta$ SRT2 vector, plasmid pSVIE was first digested with *HindIII* and *BamHI*, and the resultant *HindIII*-*BamHI* fragment was cloned into the *HindIII*-*BamHI* sites of pUC10 to generate plasmid pUIE. pUIE was digested with *NaeI* and self-ligated to generate plasmid pUIE $\Delta$ SRT2. Plasmid pUIE $\Delta$ SRT2 was digested with *NcoI*, and the resultant fragment was cloned into the *NcoI* site of pBR322IE. To generate mutants containing point mutations within the IE transactivation domain (TAD), PCR mutagenesis (Stratagene) was performed utilizing primers harboring the mutation of choice (Oligo's Etc. Wilsonville, OR; Integrated DNA Technologies, Inc., Coralville, IA). Mutagenized TADs were cloned into the PCR-Blunt vector (Invitrogen, Carlsbad, CA) and were sequenced in their entirety to confirm the presence of the desired mutation. Mutagenized TADs were cloned into unique *NcoI*-*NaeI* sites of the vector pIECassette. *EcoRV* and *BamHI* fragments housing various mutagenized TADs were then cloned into the *EcoRV*-*BamHI* sites of the pBR322IE vector, thereby generating a reconstituted IE ORF with the mutation of choice. The recombination vectors containing nonsense, insertion, point, or deletion mutations in the IE gene were used to generate mutant viruses that allowed domains of the IE protein essential for virus replication to be identified.

#### DNA Transfections and Production of Mutant Viruses.

RK-13 cells were transfected with various recombination vectors containing a mutant IE gene, followed by infection with the null virus KyAΔIE. Mutant viruses were thus generated by homologous recombination between the KyAΔIE viral genome and various recombination vectors containing mutant IE genes.

The recombination vector of choice was transfected into RK-13 cells as follows. RK-13 cells were plated at a density of  $2.5 \times 10^6$  cells per  $25\text{cm}^2$  in complete EMEM and permitted to attach overnight at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . The following day, RK-13 monolayers were subjected to liposome-mediated DNA transfection using Lipofectin reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. More specifically, liposomes were prepared by mixing 20 $\mu\text{l}$  Lipofectin reagent with 230 $\mu\text{l}$  serum-free EMEM and incubating the mixture at room temperature for 45 min. Up to 10 $\mu\text{g}$  DNA prepared in a separate tube containing 250 $\mu\text{l}$  serum-free EMEM was gently mixed into the preformed liposome solution. The liposome-DNA mixture was incubated at room temperature for an additional 15 min, and the RK-13 cell monolayers were washed three times with serum-free EMEM. The liposome-DNA solution was added drop-wise to each RK-13 cell monolayer containing 1 ml serum-free EMEM. The cells were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 5 h. Five to ten ml of EMEM supplemented with 5% FBS were added to transfected cells after 5 h, and the cells were further cultured at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ .

At approximately 24 h post-transfection, cells were superinfected at an MOI of 10 with KyAΔIE. Cells were monitored for cytopathic effects (CPE), and culture supernatants containing putative recombinant virus were plated

in 10-fold dilutions on confluent IE13.1 cells. IE13.1 cells were overlaid with 2% agarose mixed in a 1:1 ratio with 2X EMEM supplemented with 6% FBS and 1 mM BlueGal (Life Technologies) to distinguish between white and blue plaques. 5 White plaques were picked and subjected to five rounds of plaque purification on IE13.1 cells to ensure the removal of residual KyAAIE.

#### PCR analyses of virus isolates.

10 Mutant viruses isolated from plaque-purified stocks were subjected to both PCR and DNA sequence analyses using the lacZ- or IE-specific to confirm that both IE ORFs of each mutant virus harbored the desired mutation.

Viruses were isolated from culture supernatants by 15 ultracentrifugation in an SW50.1 rotor at 28,000 rpm for 2 h at 4°C. Supernatants were aspirated, and the remaining pellet was resuspended in 50 µl of RIPA buffer (150mM NaCl, 50mM Tris-HCl [pH 8.0], 0.1% SDS, 0.5% deoxycholate, 1% NP40) and incubated at room temperature for 1 h with occasional mixing. 20 Resuspended pellets served as templates for PCR analyses. IE-specific DNA fragments were amplified using an IE forward primer (5'CCTTCCCTTCTCGGTCTT3' (SEQ ID NO: 3); nt 913-930) and an IE reverse primer (5'CTCCACCCCGAACATGTT3' (SEQ ID NO: 4); nt 1155-1138). The lacZ-specific DNA fragments were amplified 25 using the IE forward primer (above) and a lacZ-specific primer (5'GGGTAACGCCAGGGTTTT3' (SEQ ID NO: 5)) derived from the 5' coding region of the *E. coli* lacZ gene. The mutations were confirmed by sequence analyses using Promega's *fmol* Sequencing System (Madison, WI).

In the case of the mutant viruses, it was found that both copies of the bacterial *lacZ* ORF of KyAΔIE parent virus were replaced with the mutated IE ORF derived from the recombination vectors.

5

### Example 3

#### Expression of Mutant IE Proteins in Infected Cells

Western blot analyses were performed to confirm that  
5 the IE protein was produced in infected cells and was of the  
predicted size. These analyses were especially important for  
mutant viruses containing large deletions in the IE gene since  
these deletions could result in the synthesis of IE proteins  
that are highly unstable.

10 Infected cell extracts were prepared according to  
the protocol described by Garko-Buczynski et al. (Virology  
248: 83-94, 1998).

Nuclear extracts of EHV-1 infected cells were  
prepared by a modification of the procedures described by  
15 Paterson et al. (Nuc. Acids. Res. 16:11005-11025, 1988). RK-  
13 cells were infected with wild-type EHV-1 or mutant EHV-1 at  
an MOI of 10. Six h post-infection, cells were scraped into  
PBS, pelleted, and resuspended into 4 volumes of buffer A (10  
mM HEPES [pH 7.0], 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.5%  
20 NP40, and 0.5 mM TPCK). Cells were incubated 10 min on ice,  
and the nuclei were pelleted at 14,000 rpm for 5 min in a  
microcentrifuge. The supernatant was discarded, and the  
nuclei were resuspended in 2 volumes of buffer B (20 mM HEPES  
[pH 7.9], 25% glycerol, 0.42 M NaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl,  
25 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM TPCK) and incubated on  
ice for 30 min. Nuclear debris was pelleted by centrifugation  
at 14,000 rpm for 15 min in a microcentrifuge, and  
supernatants were stored at -80°C.

Total protein in cell or nuclear extracts was  
30 determined by use of the bicinchoninic acid (BCA) protein  
assay reagent (Pierce, Rockford, IL). Protein samples of

infected cell extracts were boiled for 5 min with an equal volume of 2X Laemmli sample buffer (10% 2-mercaptoethanol, 4% SDS, 20% glycerol, 120 mM Tris-HCl [pH 6.8], 0.001% bromophenol blue). Proteins were separated through a 4% stacking gel and an 8% resolving gel and were electrophoretically-transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH) at 100V for 1 h at 4°C. After transfer, the membrane was blocked for 1 h at room temperature in TBST buffer (100 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.1% Tween 20) containing 1% gelatin (Sigma). The nitrocellulose membrane was then incubated with a polyclonal anti-IE peptide-specific antiserum (1:1000 dilution), followed by incubation with goat anti-rabbit antibody (Sigma; 1:30,000 dilution) conjugated to alkaline phosphatase. This anti-IE peptide antibody was generated to a peptide spanning amino acids 425 to 445 of the IE protein and had been demonstrated to be highly reactive to the IE protein in immunofluorescence (IF) and Western blot analyses (Smith et al., Virology 202: 760-770, 1994). Each antibody was incubated in TBST containing 0.1% gelatin for 2 h with shaking, followed by three washes with TBST containing 0.1% gelatin for 15 min with shaking. The reactive proteins were visualized by incubating the membrane in NBT buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5.0 mM MgCl<sub>2</sub>, 0.33 mg/ml nitroblue tetrazolium [NBT, Gibco-BRL], and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate [BCIP] Gibco-BRL).

In the case of all 17 IE mutant viruses, the mutant IE protein was synthesized in infected RK-13 cells. Representative data for four of the IE mutant viruses are shown in Figure 3. In contrast to the wild type IE protein which migrates on SDS Page as a band at about 190-200 kDa, KyAd644/824 produced an IE protein that migrates at about 180

kDa. Mutant KyAn1029 harbors an IE ORF that lacks sequences encoding the 458 carboxyl terminal residues and thus, its IE gene product migrates at about 138 kDa. With the sole exception of the KyAΔSRT2 virus, the IE protein generated by each of the other 16 viruses migrated at the predicted molecular weight. The IE ORF of the KyAΔSRT2 virus lacks sequences encoding amino acids 88-243 and thus its predicted IE protein would migrate at approximately 179 kDa. An IE gene product of this size was detectable but at very low levels. This indicated that only very low titers of this mutant could be obtained. Alternatively, the IE protein encoded by the KyAΔSRT2 virus could be very unstable.



Example 4

## Growth of Mutant Viruses on RK-13 Cells

5           RK-13 cells and IE13.1 cells were infected with mutant viruses at a range of dilutions. Infected cells were overlaid with 2% agarose mixed in a 1:1 ratio with 2X EMEM supplemented with 6% FBS. Plaque formation was observed.

10           A summary of the growth analyses of the 17 mutant viruses on RK-13 cells and the complementing IE13.1 cell line is depicted in Table 2. Importantly, all mutant viruses were capable of replication on the IE13.1 complementing cell line. The six viruses containing point mutations within the minimal IE TAD that maps within amino acids 3-89 were able to grow on  
15 RK-13 cells. Viruses containing point mutations at highly conserved residues within the IE TAD such as at leucine 12 and at phenylalanine 15 were able to grow on RK-13 cells. Similarly, KyAL12P, which contains a leucine to proline substitution at amino acid 12 that would be expected to  
20 severely alter the secondary structure of the IE TAD, was also able to propagate on RK-13 cells. Of the mutant viruses that contain deletions within the IE ORF, KyAΔSRT1, KyAΔSRT2; KyAd178/627, and KyA552/897 were unable to grow in the absence of the wild-type IE protein produced by the

**TABLE 2**  
**Growth of IE Mutants on RK-13 and IE13.1 Cell Lines**

Virus	Mutation In IE ORF	Virus Growth <sup>a</sup>	
		RK-13 Cells	IE 13.1 Cells
WTKyA	None	Yes	Yes
KyAΔSRT1	SRT Deletion aa 181-250	No	Yes
KyAΔSRT2	SRT Deletion aa 88-243	No	Yes
KyAd178/627	DNA Binding Domain aa 178-627	No	Yes
KyAd552/897	Deleted aa 552-897	No	Yes
KyA644/824	Deleted aa 644-824	Yes	Yes
KyAn627	Nonsense Mutation @ aa 627	No	Yes
KyAn951	Nonsense Mutation @ aa 951	No	Yes
KyAn1029	Nonsense Mutation @ aa 1029	Yes	Yes
KyAn1411	Nonsense Mutation @ aa 1411	Yes	Yes
KyAln628	Insertion @ aa 628	Yes	Yes
KyAln1411	Insertion @ aa 1411	Yes	Yes
KyAD20N	Point Mutation Within the IE TAD	Yes	Yes
KyAD24N	Point Mutation Within the IE TAD	Yes	Yes
KyAL12P	Point Mutation Within the IE TAD	No	Yes
KyAL12E	Point Mutation Within the IE TAD	Yes	Yes
KyAF15D	Point Mutation Within the IE TAD	Yes	Yes
KyAE34Q	Point Mutation Within the IE TAD	Yes	Yes

<sup>a</sup> Table depicts a summary of the growth of various EHV-1 viruses harboring mutations within the IE protein propagated on RK-13 cell line and the IE protein-producing cell line, IE13.1. Viruses that have deletions or mutations within the serine-rich tract, the DNA binding domain, or the NLS failed to grow in the absence of a complementing IE protein.

IE13.1 cell line. Both KyAΔSRT1 and KyAΔSRT2 delete the entire SRT region of the IE protein, although the deletion within the IE ORF of KyAΔSRT2 (residues 88-243) is in very close proximity to the IE TAD and may affect the function and/or conformation of this critical domain. KyAd644/824 contains a deletion within region 3 of the IE protein and demonstrated no defect in growth on RK-13 cells, indicating that this region contains no domains essential for the IE protein to transactivate early genes required for virus replication. In addition to the four deletion mutants described above, mutant EHV-1 that contain nonsense mutations at amino acids 627 and 951 were not able to

replicate on RK-13 cells (both of these mutants lack the nuclear localization signal). Nonsense mutations at amino acids 1411(KyAn1411) and 1029 (KyAn1029) had no effect on the growth of these viruses on non-complementing cells. However, plaques generated by KyAn1029 were larger than those produced by wild-type KyA, and the appearance of plaques generated by KyAn1029 was delayed as compared to the wild-type KyA virus. Finally, insertion mutations within the IE ORF of KyAIn628 and KyAIn1411 had no deleterious effect on the growth or the phenotype of these mutants on RK-13 cells.

Growth curve analyses were performed to determine whether mutant viruses could grow in RK-13 cells and yield virus titers comparable to those observed with wild-type EHV-1. RK-13 cells were infected with either wild-type EHV-1 or mutant virus at an MOI of 1. Aliquots of the culture supernatants were removed at various times post-infection and analyzed by plaque assay on RK-13 cells. As shown in Figure 4, KyAIn1411, KyAIn628, and KyAd644/824 grew to levels comparable to that of wild-type EHV-1. However, the titers of KyAn1411 and KyAn1029 were significantly lower than that of wild-type virus, demonstrating that these deletions within the IE ORF impaired IE function and, ultimately, growth of the virus. This observation is consistent with the recent findings (Buczynski et al., Virus Res. 65: 131-140, 1999) that sequences within the carboxyl-terminus of the IE protein are essential for the IE protein to display maximal transactivation function.

Example 5

## Temperature Sensitivity of Mutant Viruses

To determine whether mutations within the IE ORF affect virus growth at elevated or depressed temperatures, temperature shift experiments were performed. Wild-type EHV-1 or mutant viruses were serially diluted and plated in triplicate onto RK-13 cells. Plates were incubated at 33°C, 37°C, or 39°C for five days and fixed using 10% buffered formalin (Sigma, St. Louis, MO). Following staining with methylene blue, plaques were enumerated to determine plating efficiency at the various temperatures.

Wild-type EHV-1 replicated efficiently at both 33°C and 37°C, but virus yield was reduced by approximately 70% at 39°C. Additionally, the plaque size of wild-type EHV-1 was significantly reduced at 39°C to an extent that the very small plaque size made plating efficiency difficult to calculate. However, differences between the growth of wild-type EHV-1 and mutant viruses on RK-13 cells could be established. Mutant viruses were divided into two groups based on their ability or inability to grow at 39°C (Table 3). Viruses KyAn1029, KyAIn628, KyAn1411 and KyAΔSRT2 were categorized into Group 1 based on a severe impairment of growth in RK-13 cells at 39°C as compared to growth observed for wild-type KyA. Both KyAn1029 and KyAn1411 displayed impaired growth at both at 37°C (Figure 4) and at 39°C (Table 3). Viruses categorized into Group 2 (Table 3), with the exception of KyAIn1411, grew at 39°C, although some impairment of growth was observed. KyAIn1411

was able to grow at 33°C, 37°C and 39°C to levels that appeared to be comparable to those of wild-type EHV-1.

**TABLE 3**  
**Temperature Sensitive Phenotype**

<b>Group I Mutants</b>		<b>Plaques @ 39°C<sup>a</sup></b>
KyAn1029	Nonsense Mutation @ aa 1029	None
KyAn1029	Nonsense Mutation @ aa 1029	None
KyAn11411	Nonsense Mutation @ aa 11411	None
KyAln628	Insertion @ aa 628	None
KyAΔSRT2	Deleted aa 88-243	None
<b>Group II Mutants</b>		
KyAd644/824	Deleted aa 644-824	++
KyAln1411	Insertion @ aa 1411	+++
KyAD24N	Point Mutation Within IE TAD	++
KyAL12P	Point Mutation Within IE TAD	++
KyAL12E	Point Mutation Within IE TAD	++
KyAF15D	Point Mutation Within IE TAD	++
KyAE34Q	Point Mutation Within IE TAD	++

<sup>a</sup> Viruses were divided into two groups (I and II) based on their ability or inability to grow at 39°C as compared to wild-type EHV-1. Growth at 39°C scored as comparable to wildtype EHV-1. +++ corresponds to titers that range from 10<sup>4</sup>-10<sup>5</sup>. ++ corresponds to titers that range from 10<sup>3</sup>-10<sup>4</sup>.

Example 6**Molecular Phenotype of Selected Virus Mutants**

Several viral mutants, KyAn1029, KyAn1411, KyAΔSRT2 and KyAd644/824 were selected for initial studies to define their molecular phenotype.

To characterize the defects of these mutant viruses at the level of gene expression, ribonuclease protection assays (RPA) were performed. The sensitivity of the RPA would allow identification of differences in the levels of expression of the IE gene, the EICP0 gene (an early auxiliary regulatory gene important in the expression of late genes), and the glycoprotein D (gD) gene (an important late gene essential for virus replication). RK-13 cells were mock-infected or infected with wild-type EHV-1, KyAn1029, KyAn1411, KyAΔSRT2 or KyAd644/824 at an MOI of 10. Cells were harvested at specific times (such as 3, 5, 9 hours) post-infection, and total RNA was isolated using TRIZOL REAGENT (Life Technologies) as per the manufacturer's protocol. For RPA, the RIBOQUANT RNASE PROTECTION ASSAY SYSTEM (Pharmingen, San Diego, CA) was utilized as per the manufacturer's instructions.

Approximately 20 µg of total RNA was used for each reaction of the RPA. Probes for RPA analyses were generated using Promega's (Madison, WI) RIBOPROBE IN VITRO TRANSCRIPTION SYSTEM. For IE probes, plasmid pG3IE was digested with BamHI to generate run-off transcripts. Plasmid pG3IE was constructed by cloning an NheI/DraI fragment containing the entire 4,773-bp IE ORF into the XbaI/SmaI sites of plasmid pGEM-3Z (Promega, Madison, WI). Digested pG3IE plasmid was purified by phenol-chloroform extraction and ethanol

precipitation, and approximately 1 µg of plasmid was used for riboprobe generation as per the manufacturer's instructions. For EICP0-specific probes, plasmid pGEMICP0K (Bowles et al. J. Virol. 71:4904-4914, 1997; Bowles et al., J. Virol. 74: 1200-1208, 2000) was digested with *NheI*, and run-off transcripts were generated and purified as described above. For glycoprotein D-specific probes, plasmid p72D392 (Zhang et al. Virus Res. 56:11-24, 1998) was transcribed directly using the Promega's RIBOPROBE *IN VITRO* TRANSCRIPTION SYSTEM. mRNA levels were quantitated by using the MOLECULAR DYNAMICS PHOSPHOIMAGERY SYSTEM (Sunnyvale, CA).

The levels of gene expression are summarized in Table 4. Analyses of KyAd644/824 by RPA clearly showed that gene expression at all time points was comparable to that obtained for wild-type EHV-1. These data are concordant with data obtained from growth curve analyses in that titers of KyAd644/824 were similar to those obtained for wild-type EHV-1. However, examination of the expression of these representative genes in RK-13 cells infected with KyAn1029, KyAn1411 and KyAΔSRT2 viruses revealed reduced levels of these viral transcripts especially at later time points during infection. While the expression of the IE gene in KyAn1029-infected cells was unaffected at both 3 and 5 h post-infection, the level of IE mRNA at 9 h post-infection was reduced almost three-fold. Furthermore, EICP0 mRNA synthesis was reduced at all time points tested in KyAn1029-infected cells, suggesting again that the carboxyl-terminus of the IE protein is important for full activity of this regulatory protein. The reduction in EICP0 gene expression also significantly



impacted late gene expression, as seen from the gD mRNA synthesis which was reduced by more than three-fold at late times during infection. Analyses of KyAΔSRT2 gene expression by RPA clearly demonstrated that deletion of the SRT region did not affect IE gene expression, but both EICPO and gD gene expression was reduced to levels barely detectable at 9 h post-infection. Defects in both early and late gene expression irrevocably impaired the virus, thereby making the KyAΔSRT2 virus unable to propagate in RK-13 cells.

**TABLE 4**  
**Summary of RPA Analyses**

Virus Mutant	%KyA WT mRNA Expression <sup>a</sup>		
	3hr p.i.	5hr p.i.	9hr p.i.
<b>KyAd644/824</b>			
IE	98%	99%	97%
EICPO	99%	92%	95%
gD	_b	98%	89%
<b>KyAn1029</b>			
IE	99%	92%	34%
EICPO	46%	57%	36%
gD	_b	78%	29%
<b>KyAn1411</b>			
IE	98%	97%	98%
EICPO	98%	88%	86%
gD	_b	85%	80%
<b>KyAΔSRT2</b>			
IE	98%	97%	92%
EICPO	99%	42%	3%
gD	_b	7%	5%

<sup>a</sup> Values shown are depicted as the percent mRNA detected as compared to those obtained for wild-type EHV-1 IE, EICPO, and gD mRNA expression.

<sup>b</sup> gD is a late gene and no gD transcript was detected at immediate-early times in cells infected with wild-type virus or any of the mutant viruses.

### Example 7

#### Testing Mutant Viruses in Mice

Mutant viruses KyAd644/824, KyAn1411, KyAin1411 and KyAE34Q were tested in mice.

Female CBA (H-2k) mice, 3 to 6 weeks of age, were obtained from Harlan Sprague Dawley, Indianapolis, Ind., or Jackson Laboratory Bar Harbor, Maine. Mice were maintained in the Animal Resource Facility of the Louisiana State University Medical Center, Shreveport, in cages equipped with filter tops. All mice were rested for a minimum of 1 week prior to use.

Mice were anesthetized with Halothane (Sigma Chemical Co., St. Louis, Mo.) and inoculated intranasally (i.n.) with  $2 \times 10^6$  PFU of EHV-1 KyA or a mutant virus in a volume of 50  $\mu$ l. Control mice received 50  $\mu$ l of culture medium alone.

#### Attenuation

Immunized mice were monitored daily for development of clinical signs of EHV-1 infection such as ruffled fur, loss of body weight, labored breathing, lethargy and huddling, as described by, e.g., Colle et al. Virus Res. 43: 111-124 (1996) and Zhang et al. Virus Res. 56: 11-24 (1998). No clinical disease was observed with mice infected with any of the four mutant viruses tested.

In addition, the levels of virus present in the lungs on days 2, 5 and 8 post-innoculation were determined by plaque titration on RK-13 cell monolayers. Virus were isolated from the lung as described by Colle et al. (1996) and by Smith et al. J. Virol. 72: 5366-5372 (1998).

### CTL Response

To assess primary CTL responses, lymphocytes were isolated from the mediastinal lymph nodes (MLN) 5 days postinoculation, and a single-cell suspension was obtained by pressing the lymphoid tissues through a 60-gauge wire mesh screen. The lymphocytes were washed and cultured ( $10^7$  cells per well) for 3 days at 37°C and 5% CO<sub>2</sub> in 12-well flat-bottom plates (Corning Inc., Corning, N.Y.) in complete RPMI 1640 (Sigma) containing 5% FCS, 20  $\mu$ M - mercaptoethanol, 20 mM HEPES, 2 mM L-glutamine and antibiotics. Cytolytic activity was assessed in a standard 4-h <sup>51</sup>Cr release assay in 96-well V-bottom plates (Nunc, Denmark) at a range of effector-to-target ratios against  $10^4$  <sup>51</sup>Cr-labeled, infected or uninfected target LM cells.

As indicated in Figures 5A-5C, all four mutant viruses tested, KyAd644/824, KyAn1411, KyAin1411 and KyAE34Q, induced a CTL response at a level similar to that induced by parent KyA virus .

To assess memory CTL responses, mice were infected as described above and maintained for 2-26 weeks. Spleen tissues were pressed through a 60-gauge wire mesh screen, and the cell suspension was subjected to a brief exposure at 37°C to Tris-buffered 0.83 NH<sub>4</sub>Cl to lyse erythrocytes. The resulting lymphocytes were then cultured ( $10^7$  cells per well) in complete RPMI 1640 at 37°C and 5% CO<sub>2</sub>, for 5 days in 12-well flat-bottom plates in the presence of  $3 \times 10^5$  mitomycin C-treated stimulator cells, as described by Jennings et al. Cell. Immunol. 133: 234-252 (1991). The stimulator cells were L-M mouse fibroblasts cells that had been infected with EHV-1 KyA at a multiplicity of infection of 10 for 18 h to allow the expression of late viral gene products. Cytolytic activity

was assessed in a standard 4-h  $^{51}\text{Cr}$  release assay in 96-well V-bottom plates (Nunc, Denmark) against  $10^4$   $^{51}\text{Cr}$ -labeled, infected or uninfected LM cells.

#### **EHV-1 Specific Antibody in the Serum of Immunized Mice**

Serum samples from immunized and control mice were assayed for the presence of neutralizing antibody (nAb). Briefly, after the sera were heated to  $56^\circ\text{C}$  for 1 h to inactivate complement, serial, two-fold serum dilutions (1:20 to 1:1028) were incubated with  $3 \times 10^3$  pfu EHV-1 KyA for 1 h at  $37^\circ\text{C}$ . Each sample was assayed in triplicate for infectious virus by plaque assay on RK-13 cell monolayers. The nAb titer was determined as the reciprocal of the highest dilution of serum resulting in a 50% reduction in pfu. Preimmune serum was employed as a control.

#### **EHV-Specific Antibody Secreting Cells (ASC)**

On specified days postchallenge, the mice were terminated, and the lungs, MLN, and CLN were removed, and the spectrum of ASC was determined by ELISPOT assay. To generate a single-cell suspension from the lung tissue, the tissue was fragmented with scissors and then pressed through a 60-gauge screen. After centrifugation, the cells were resuspended in 10 ml collagenase type L/deoxyribonuclease L solution (250:50 U/ml; Gibco-BRL) and incubated in a shaker at  $37^\circ\text{C}$  for 90 min. The cells were then treated with 3 ml of Tris-buffered 0.83%  $\text{NH}_4\text{Cl}$  solution for 5 min at  $37^\circ\text{C}$  to lyse red blood cells. The cells were resuspended in Dulbecco's modified Eagle's medium, supplement with 5% calf serum and antibiotics (DMEM-5). To generate a single-cell suspension from MLN and CLN, the tissues were pressed through a 60-gauge screen without

additional treatment. To generate a single-cell suspension from spleens, splenic tissue was pressed through a 60-gauge screen and treated with Tris-NH<sub>4</sub>CL, as before.

**ELISPOT assay.** Briefly, nitrocellulose-based microtiter 96-well plates (Millititer-HA, Millipore Corporation, Bedford, MA) were coated with EHV-1 KyA-infected cell lysate (10-200 µg/ml) in phosphate-buffered saline (PBS) and incubated in humidified chambers at 4°C overnight, or at 37°C for 2 h. Coated plates were stored at 4°C until use. For each experimental determination, the coating buffer (PBS) was decanted and the plates were washed three times by rinsing with PBS. Potential remaining binding sites were saturated with 5% fetal calf serum in PBS or 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in PBS for 30 min at 37°C. Following additional washes, the lymphocytes, diluted to the appropriate concentration (10<sup>3</sup> to 10<sup>6</sup> cells/well) in DMEM-5, were added to the coated plates. Lipopolysaccharide (LPS, 5 µg/well; Calbiochem-Novabiochem, La Jolla, CA) was added as a nonspecific activator of B cells. A control plate of uninfected cells was also used for the assays and was treated in the same manner as the plates of EHV-1 infected cells.

The cells were incubated for 3 to 4 h at 37°C in 5% CO<sub>2</sub> and then were discarded by washing with PBS containing 0.005% Tween 20 (PBST). One hundred microliters of alkaline phosphatase-conjugated antibody was added to each well, and the plates were incubated for 2 to 3 h at room temperature in humidified chambers. After thorough washing, the spots corresponding to EHV-1-specific ASC were visualized by adding 5-bromo-4chloro-3-indolyphosphate p-toluidine salt (BCIP, Gibco-BRL) and Nitroblue tetrazolium

chloride (NBT, Gibco-BRL) as substrate, according to the manufacturer's instructions, for 5 to 30 min until the blue color developed. The nitrocellulose membranes were washed for a few seconds in water to stop the reaction. After 2 to 24 h, EHV-1-specific ASC were counted under low (10-30X) magnification in a light microscope.

#### Induction of Protective Immunity

Immunized mice were lightly anesthetized with Halothane (Halocarbon Laboratories, River Edge, NJ) and then inoculated intranasally with a pathogenic EHV-1 strain at  $2 \times 10^6$  pfu diluted into PBS (50  $\mu$ l total volume/mouse).

Mice were monitored daily for the development of clinical signs of EHV-1 infection such as ruffled fur, loss of body weight, labored breathing, lethargy and huddling.

In addition, the level of virus in the lungs of challenged mice were determined. Lungs from the challenged animals were isolated at days 2, 5 and 10 post-challenge. Virus was recovered from mouse lung tissue as described previously by Awan, et al. J. Gen. Virol. 71: 1131-1140 (1990). Tissues were homogenized in 1 ml Eagles minimal essential medium using a 2 ml Wheaton Dounce homogenizer. Each sample was then sonicated in an Ultrasonic processor (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) for one minute and then centrifuged at 3,000 rpm for ten minutes to remove cell debris. Samples were stored at -70°C until virus was quantitated by plaque assay on RK cells. Statistical analyses were done using the SigmaStat software (Jandel, Corp., San Rafael, CA).

SEQ ID NO: 1 (Equine herpesvirus type 1 immediate-early gene,  
GENEBANK Accession number J04366)

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SEQ ID NO: 2 (Equine herpesvirus type 1 immediate-early protein,  
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1261 farlypdsap lrlcrssnva ytvdrager trvplaprey rqr vlpdydg ckdmraqaeg
1321 lgfhdpdfee gaaqshraan rwglgawlrp vylacgrrga gavepselli pellsefcrv
1381 allepd aeae plvlpiteap rrraprvdwe pgfgrstsv lhmgatecll pepddeleid
1441 gagdvelvve hpgpspgvaq alrrapikie vvsddedggd wcnpyls

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SEQ ID NO: 3 5' CCTTCCCTTCTCGGTCTT3'

SEQ ID NO: 4 5' CTCACCCCGAACATGTT3'

SEQ ID NO: 5 5' GGGTAACGCCAGGGTTT3'

## We claim:

1. An EHV-1 isolate comprising a mutation selected from Table 1.
2. The EHV-1 isolate of claim 1, wherein said EHV-1 isolate is of a strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.
3. An EHV-1 isolate selected from the group consisting of KyAASRT1, KyAASRT2, KyAd178/627, KyA552/897, KyA644/824, KyAn627, KyAn951, KyAn1029, KyAn1411, KyAin628, KyAin1411, KyAD20N, KyAD24N, KyAL12P, KynL12E, KyAF15D and KyAE34Q.
4. A replication-competent EHV-1 isolate comprising a mutation in the IE gene.
5. The EHV-1 isolate of claim 4, wherein said mutation is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.
6. The EHV-1 isolate of claim 5, wherein said substitution is selected from D20N, D24N, L12E, F15D or E34Q.
7. The EHV-1 isolate of claim 4, wherein said mutation is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

8. The EHV-1 isolate of claim 7, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.
9. The EHV-1 isolate of claim 4, wherein said EHV-1 isolate is non-pathogenic.
10. The EHV-1 isolate of claim 9, wherein said EHV-1 isolate is of a strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.
11. The EHV-1 isolate of claim 10, wherein the mutation in the IE gene of said EHV-1 isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.
12. The EHV-1 isolate of claim 11, wherein said mutation is selected from D20N, D24N, L12E, F15D or E34Q.
13. The EHV-1 isolate of claim 12, selected from KyAD20N, KyAD24N, KyAL12E, KyAF15D or KyAE34Q.
14. The EHV-1 isolate of claim 10, wherein the mutation in the IE gene of said EHV-1 isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.
15. The EHV-1 isolate of claim 14, wherein said isolate is selected from d644/824, n627, n1029, n1411, in628 or in1411.

16. The EHV-1 isolate of claim 15 selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.
17. A immunogenic composition comprising a pharmaceutical-acceptable carrier and a nonpathogenic, replication-competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.
18. The immunogenic composition of claim 17, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.
19. The immunogenic composition of claim 18, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.
20. The immunogenic composition of claim 19, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.
21. The immunogenic composition of claim 20, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.
22. The immunogenic composition of claim 18, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three

amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

23. The immunogenic composition of claim 22, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.
24. The immunogenic composition of claim 23, wherein said isolate is selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.
25. A method of stimulating an immune response against EHV-1 in a horse subject, comprising administering to said subject an effective amount of a nonpathogenic, replication-competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.
26. The method of claim 25, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.
27. The method of claim 26, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.
28. The method of claim 27, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.



29. The method of claim 28, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.
30. The method of claim 26, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.
31. The method of claim 30, wherein said mutation is selected from  $\Delta$ 544/824, n627, n1029, n1411, in528 or in1411.
32. The method of claim 31, wherein said isolate is selected from KyAd544/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.
33. The method of claim 25, wherein said immune response is a cell-mediated immune response or a humoral immune response.
34. An vaccine composition comprising a pharmaceutical-acceptable carrier and a nonpathogenic, replication-competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.
35. The vaccine composition of claim 34, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.

36. The vaccine composition of claim 35, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.
37. The vaccine composition of claim 36, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.
38. The vaccine composition of claim 37, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.
39. The vaccine composition of claim 35, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1437.
40. The vaccine composition of claim 39, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.
41. The vaccine composition of claim 40, wherein said isolate is selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.
42. A method of preventing or inhibiting an EHV-1 infection in a horse subject, comprising administering to said subject a therapeutically effective amount of a nonpathogenic, replication-

competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.

43. The method of claim 42, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.
44. The method of claim 43, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.
45. The method of claim 44, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.
46. The method of claim 45, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.
47. The method of claim 43, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.
48. The method of claim 47, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.
49. The method of claim 48, wherein said isolate is selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.

50. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene, said method comprising isolating said virus from said subject, detecting the presence of the mutant IE protein of said non-pathogenic isolate and the absence of a wild type IE protein in said virus, thereby determining said virus as non-pathogenic.
51. The method of claim 50, wherein the mutant IE protein has a mobility on SDS PAGE different from that of a wild type IE protein.
52. The method of claim 51, wherein the mutant IE protein comprises a deletion or insertion of amino acid residues, and wherein said detection of the IE protein is based on an antibody specific for the deleted or inserted amino acid residues.
53. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a deletion in the IE gene, said method comprising detecting the absence in the serum of said subject of an antibody specific for the deleted portion of the IE protein, thereby determining said virus as non-pathogenic.

54. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene, said method comprising isolating said virus from said subject, detecting the absence of the wild type IE nucleotide sequence and the presence of the mutant IE nucleotide sequence, thereby determining said virus as non-pathogenic.
55. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene, said method comprising isolating said virus from said subject, determining the temperature sensitivity of said virus as identical to that of said non-pathogenic EHV-1 isolate, thereby determining said virus as non-pathogenic.

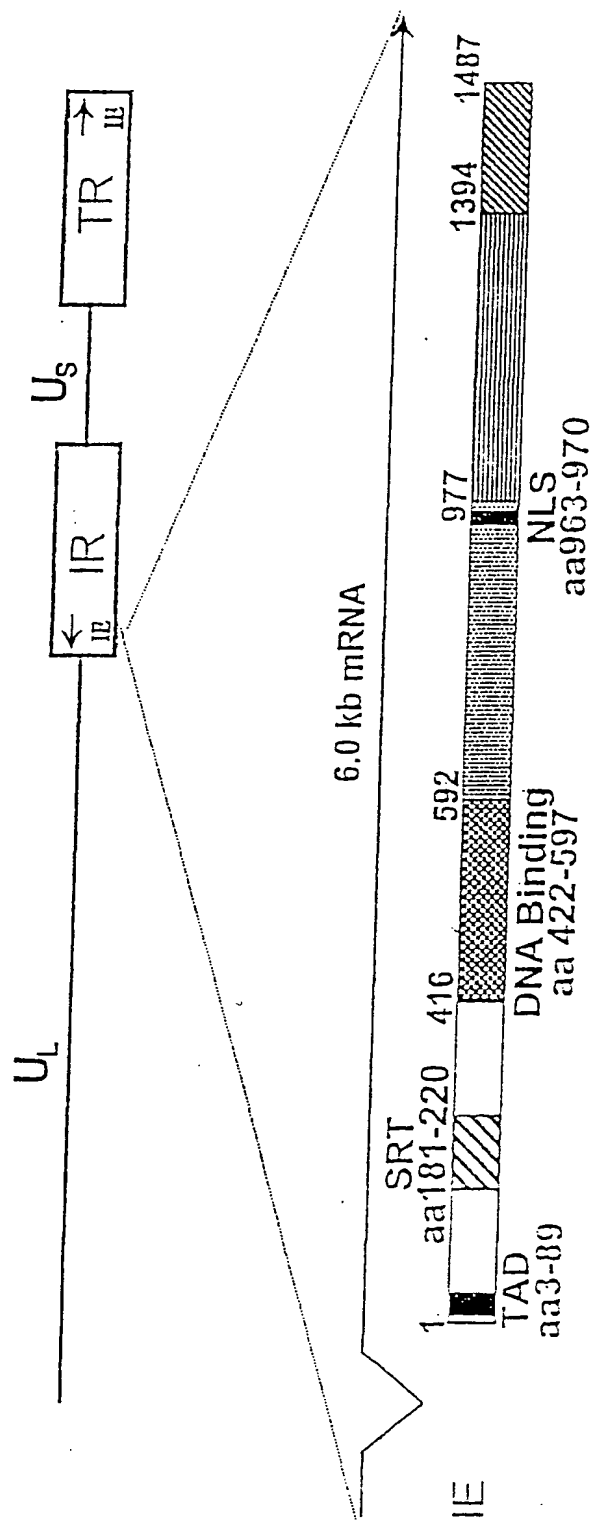


Fig. 1

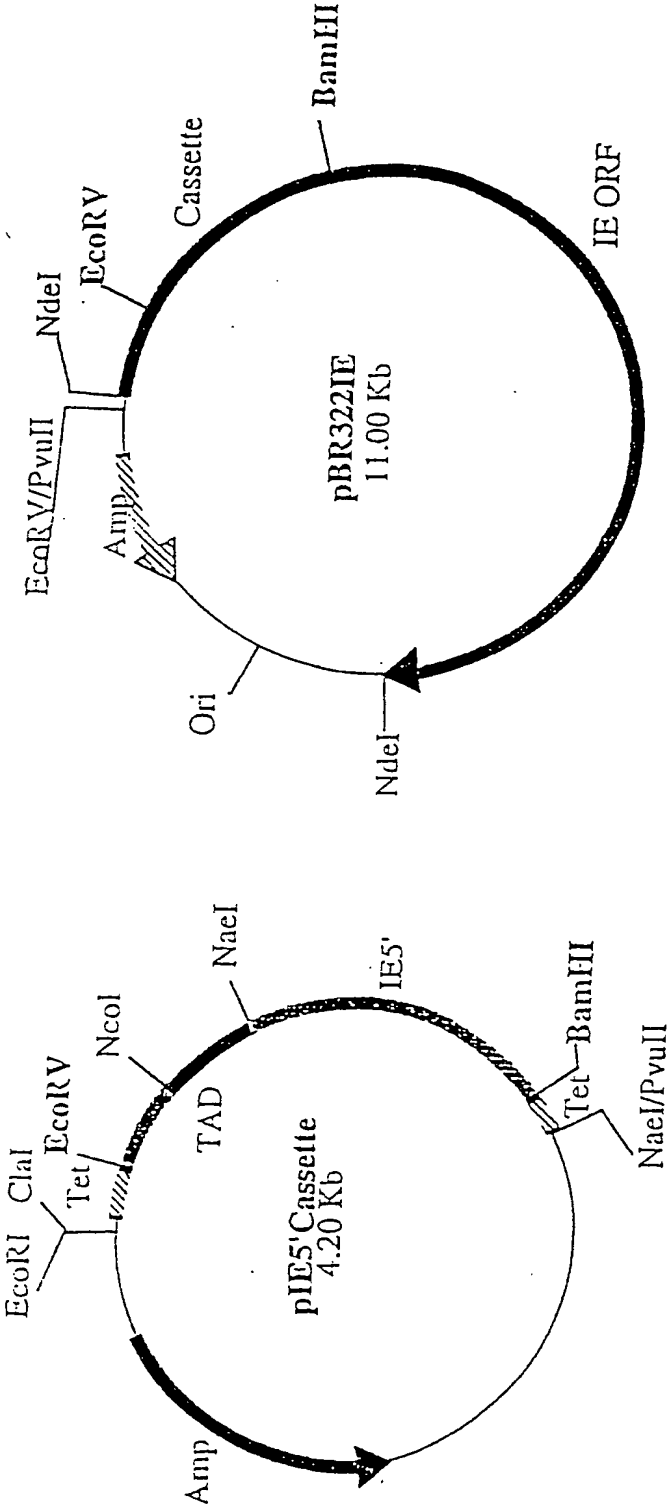


Fig. 2

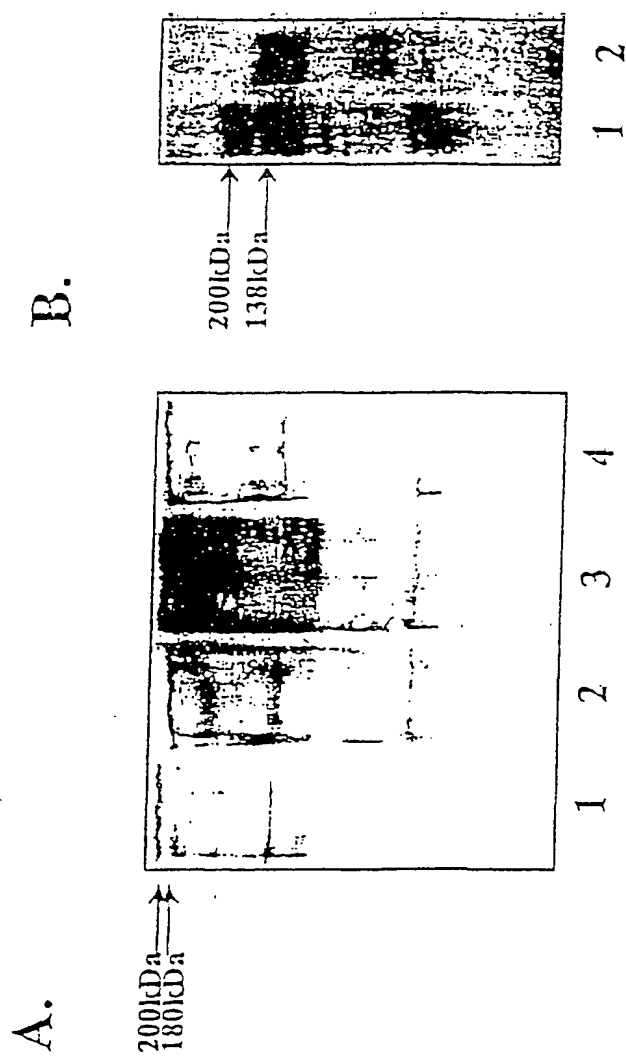


Fig. 3



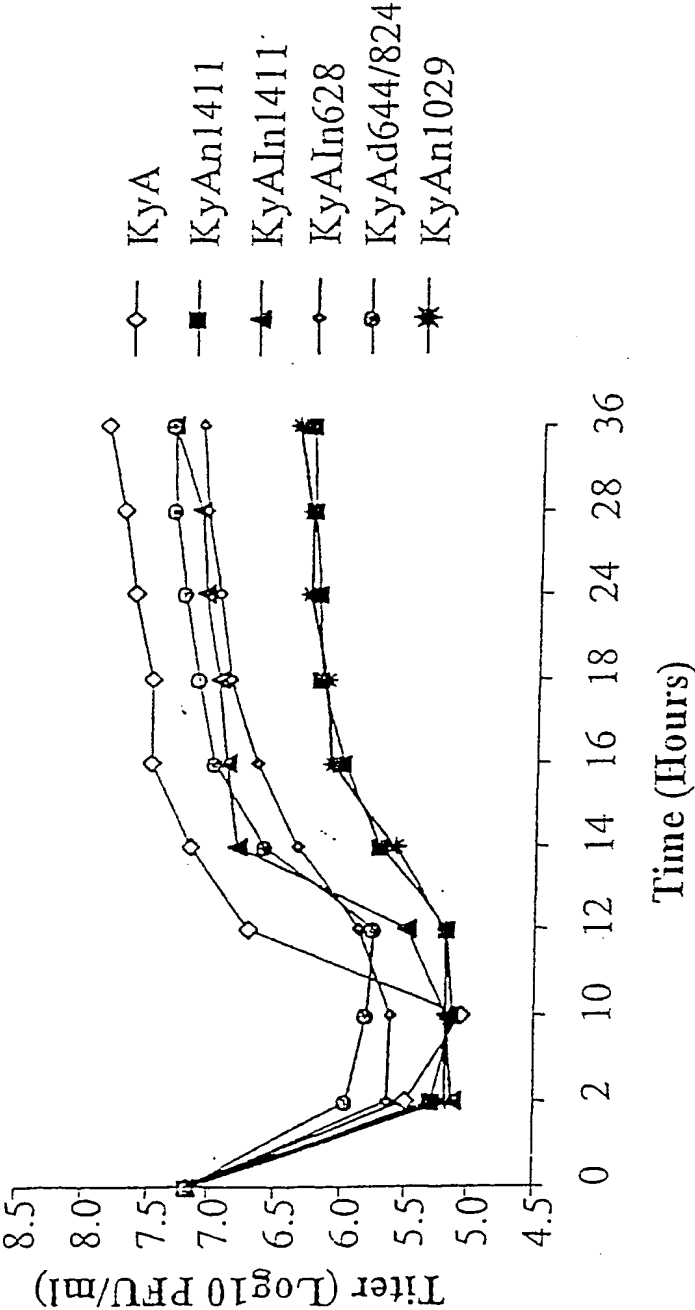


Fig. 4

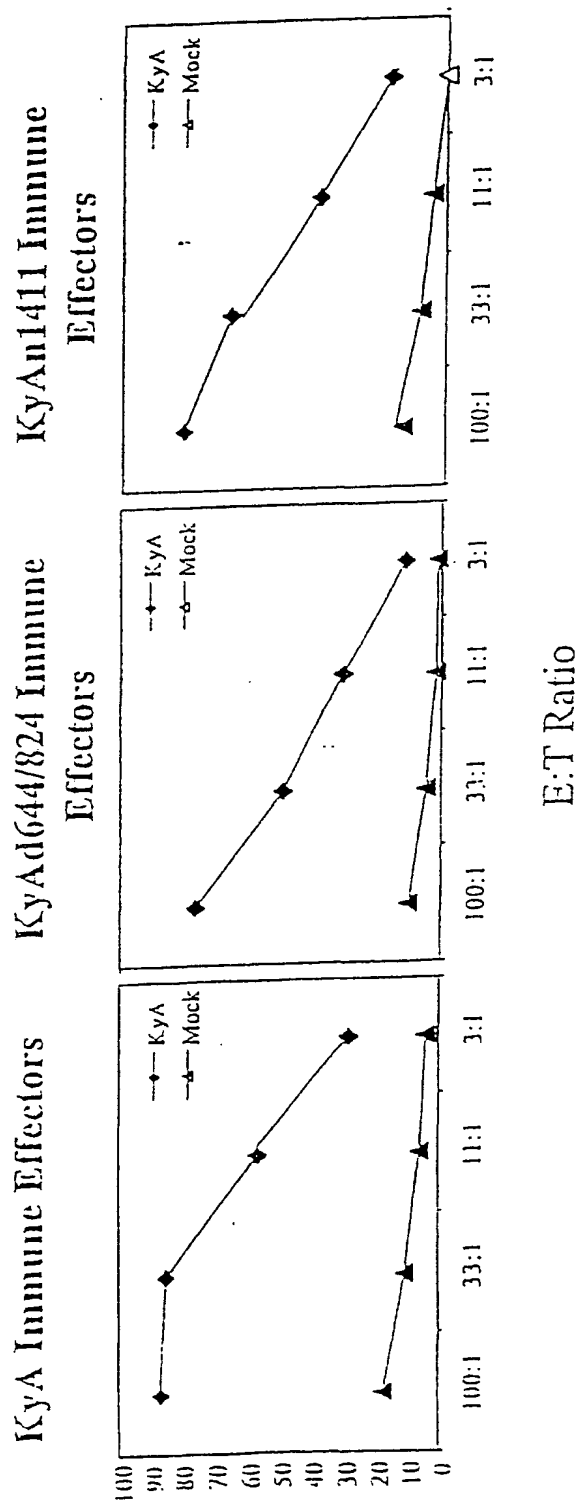


Fig. 5A

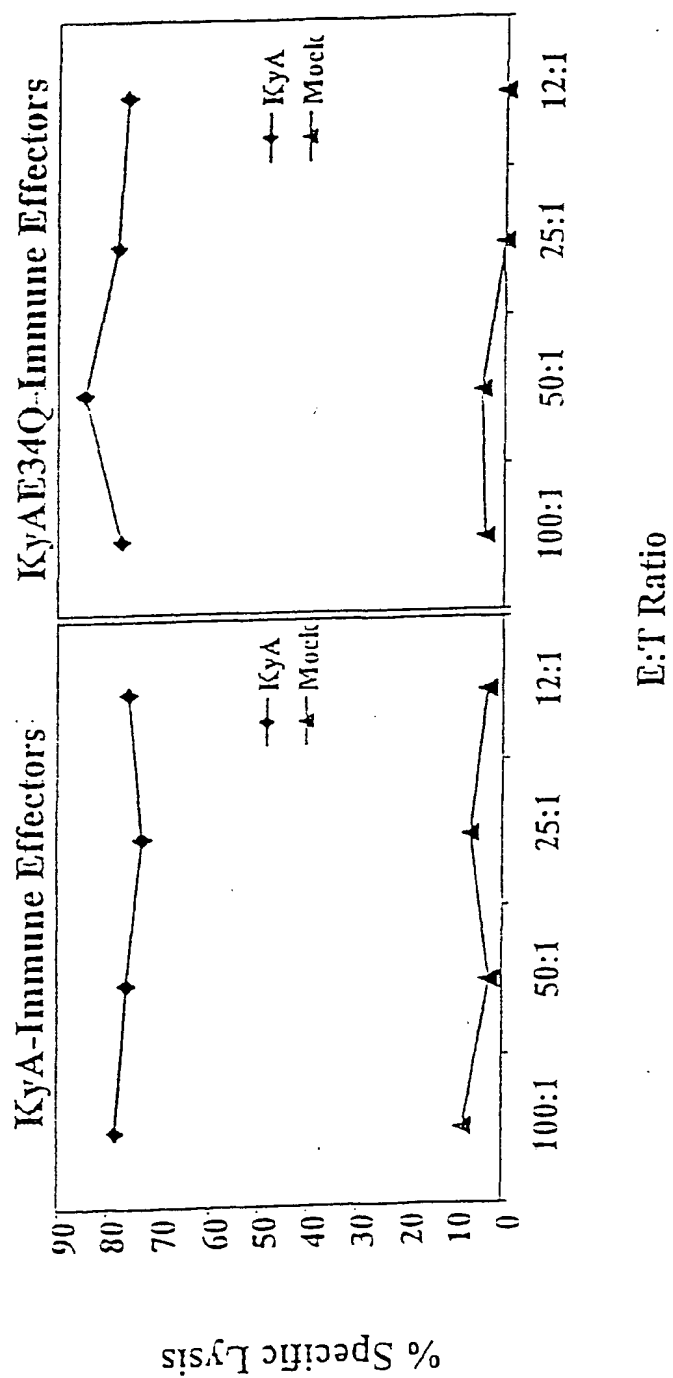


Fig. 5B

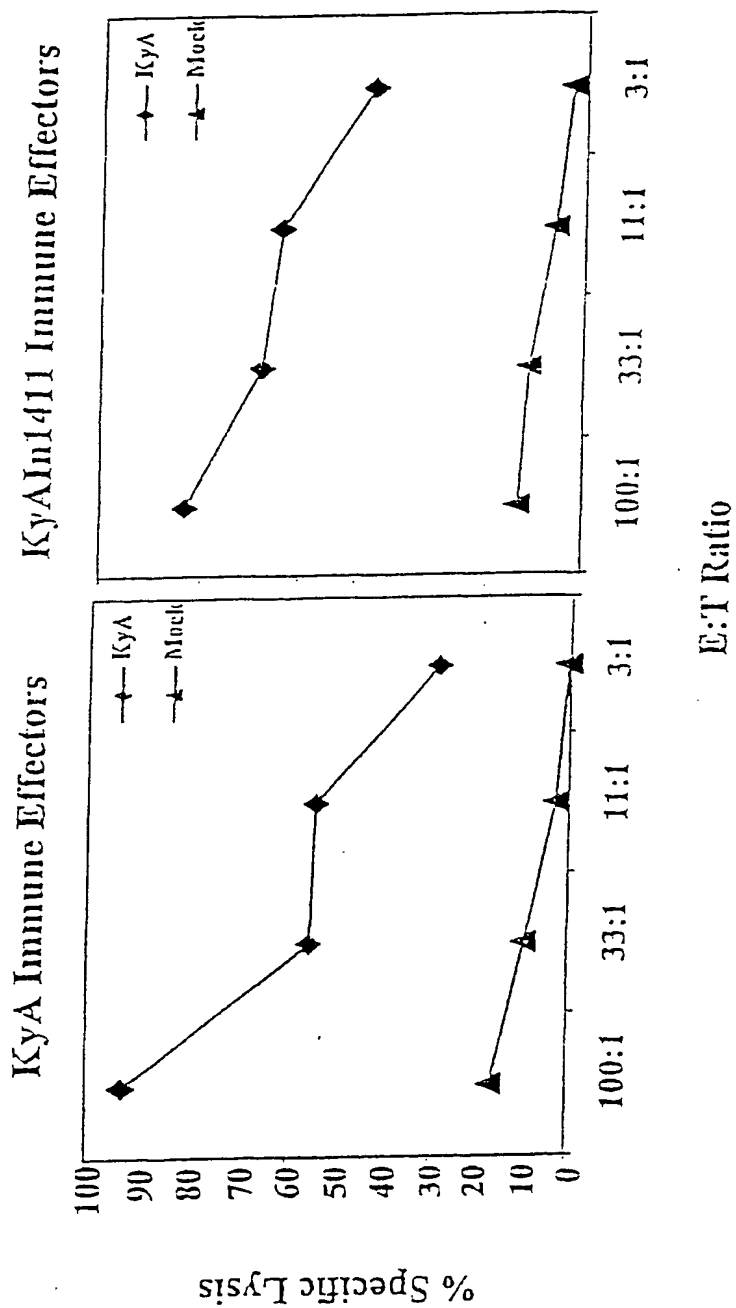


Fig. 5C